

Effects of the sublethal doses of imidacloprid on the bacterial diversity in the midgut of *Apis mellifera ligustica* (Hymenoptera: Apidae)

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Abstract: 【Aim】 In this study, the potential side effects of the sublethal doses of imidacloprid on the midgut bacteria of the worker bees of *Apis mellifera ligustica* was investigated under laboratory conditions so as to provide a theoretical basis for the biosafety assessment of pesticides. 【Methods】 Newly emerged bees were fed on syrups with different sublethal doses of imidacloprid (0.005 mg/L, 0.015 mg/L and 0.045 mg/L) and pure sugar syrup with 0.3% acetone, and samples were collected after 3, 6, 9, 12 and 15 d, respectively. The population structure of bacteria in the midgut of honeybees in the four treatment groups was analyzed using next-generation sequencing after the genomic DNA was gained from intestinal bacteria. 【Results】 The results of sequence analysis showed that the midgut bacteria in *A. mellifera ligustica* mainly belong to Proteobacteria and Firmicutes. The composition of dominant midgut bacteria and the richness estimators in the same period were analyzed for statistical significance among the four treatments by performing a *T*-test, and no significant differences were observed ($P > 0.05$). 【Conclusion】 The results suggest that the sublethal doses of imidacloprid does not induce significant changes in the composition of the honeybee gut bacterial community under laboratory conditions.

Key words: *Apis mellifera ligustica*; biosafety; imidacloprid; sublethal effect; intestinal community; next-generation sequencing

1 INTRODUCTION

Neonicotinoids are a group of the most broadly used insecticides with more than 25% share of global market (Van der Sluijs *et al.*, 2013). Imidacloprid is a representative of neonicotinoid insecticide. Due to its high efficiency, low toxicity, and broad-spectrum, imidacloprid has become the second most used agrochemical in the world.

Honey bees are the most important pollinators in natural ecosystems world-wide, and play an important role in biodiversity maintenance and ecosystem function. They, therefore, have been widely considered as an important insect in the biosafety assessment of insecticides. The population number of honeybees has declined in recent years (Brown and Paxton, 2009; Cameron *et al.*, 2011). Many investigators suspected that extensive pesticide use is one of the major contributors to heavy bee losses worldwide (vanEngelsdorp and Meixner, 2010; Blacquiere *et al.*, 2012). In May 2013, the

European Commission (EC) adopted a 2-year restriction to the use of three neonicotinoid pesticides (imidacloprid, clothianidin, and thiamethoxam) on crops that attract bees and other pollinators in an effort to reduce bee losses and to re-evaluate the risk of these pesticides on honeybees within the two years (Maxim and Arnold, 2013).

Many environmental factors affect bees, but do not cause direct mortality. Those factors are called sublethal impact. Honeybees tend to be exposed to pesticides at sublethal doses in the real situation, so assessment of sublethal effects of pesticides on bees has been a subject with increasing discussion within scientists (Laurino *et al.*, 2011). Sublethal effect of pesticides on honeybees has been most widely studied with the focus mainly on foraging behavior (Decourtye and Devillers, 2010), learning performance and memory (Decourtye *et al.*, 2011), motor activity (Lambin *et al.*, 2001), and olfactory capacity (Hassani *et al.*, 2005).

A number of findings are revealing that there is a strict interconnection between the intestinal

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microbiota balance and the health status of the honeybees (Cox-Foster *et al.*, 2007; Fraune and Bosch, 2010; Engel *et al.*, 2012). The possibility of using the gut bacterial community as an indicator of honeybee health has been discussed (Crotti *et al.*, 2013), but there are no reports focusing on the midgut bacterial structure in honeybees exposed to imidacloprid. The aim of the present study is to investigate the impact of imidacloprid, administered at sublethal doses, on the midgut bacterial community of worker bees over time under laboratory conditions.

2 MATERIALS AND METHODS

2.1 Insect and sublethal doses of imidacloprid preparation

Newly emerged worker bees (less than 1 h old) were chosen in the experiment; the bees were randomly sampled from the apiaries of Department of Bee Protection and Biological Safety, Institute of Apicultural Research, Chinese Academy of Agricultural Sciences, Beijing, China.

Imidacloprid (99.99% pure) obtained in powdered form from Sigma-Aldrich (Germany). To identify whether the concentration of imidacloprid influenced the intestinal bacterial community of the honeybees, three concentrations (0.005, 0.015, and 0.045 mg/L) of imidacloprid were used in the experiment. A stock solution of imidacloprid was prepared in acetone. Aliquots of the stock solution were used to make each test solution at a specific concentration, which were added to sugar syrup [50% (w/v) sucrose solution] to obtain the desired imidacloprid concentrations. The final concentration of acetone in the sucrose solutions was 0.3%. The effects of imidacloprid-containing solutions were compared with the control group that received the treatment of sucrose solution containing 0.3% acetone.

2.2 Laboratory feeding of honeybees

Newly emerged bees (less than 1 h old) were assigned randomly to wooden cages (dimensions of 10 cm × 7 cm × 8 cm) with mesh on two sides. For each treatment, a total of 80 bees were used. All the cages were kept in a dark incubator at 29 ± 1°C and 65% ± 5% relative humidities. Newly emerged workers possess few bacteria (Martinson *et al.*, 2012), so bees were fed with 2 000 µL of sugar syrup [50% (w/v) sucrose solution], 300 µL of water *ad libitum*, and 0.5 g of pollen from a gravity feeder fitted on each cage for the first 7 days (Ramirez-Romero *et al.*, 2008; Dai *et al.*, 2012). From the eighth day, the laboratory honeybees were exposed to sublethal doses of imidacloprid, and 33 µL of the sugar solution per bee was supplied every

day (Decourtye *et al.*, 2003). Four treatments were designed to examine the effects of the sublethal doses of imidacloprid on the intestinal bacterial community of the honeybees, and for each treatment, three replicates were undertaken. A group of bees receiving the treatment of sucrose solution containing 0.3% acetone is served as a negative control group. Honeybees of the other three treatments were fed on syrups with 0.005 mg/L, 0.015 mg/L, and 0.045 mg/L of imidacloprid, respectively. Bees were collected for further analysis following exposure to imidacloprid for 3, 6, 9, 12 and 15 d, respectively.

2.3 Honeybee dissection and DNA extraction

The honeybees were dissected at 3, 6, 9, 12 and 15 d after treatment, respectively. The bees were placed at -20°C for 20 s before dissection. The midguts of the honeybees were isolated in sterile conditions, and ten samples were collected for each treatment, kept in 2.0 mL eppendorf tubes and immediately stored at -70°C until use. DNA extraction was performed according to the QIAamp DNA Stool Mini Kit protocol (QIAGEN, Germany).

2.4 16S rRNA gene V3 region PCR and pyrosequencing

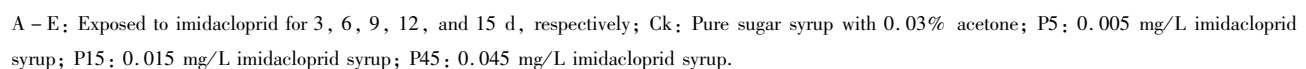
Amplification of partial bacterial V3 region of 16S rRNA gene sequences were performed as previously described (Nakatsu *et al.*, 2000) with the primer pair V3-343f and V3-534r. PCR reactions were performed in a volume of 50 µL containing 22 µL sterile water, 1.0 µL DNA template, 1.0 µL of each primer, and 25 µL 2 × Master Mix (Promega, USA). The following touchdown PCR protocol was used: initial denaturation at 94°C for 5 min, followed by 10 cycles at 94°C for 30 s, 60°C for 45 s, and 72°C for 90 s, in which the annealing temperature was reduced by 0.5°C/cycle from the preceding cycle, followed by 27 cycles of 94°C for 30 s, 55°C for 45 s, and 72°C for 90 s. The PCR products (about 200 bp) were separated by electrophoresis on agarose gels (1.5%, w/v) and recovered with a DNA Gel Extraction Kit (AxyGEN, USA). The amplified PCR products were sequenced by the Illumina HiSeq 2500 according to the manufacturer's instructions in Beijing Computer Center (Beijing, China).

2.5 Sequence Analysis

Illumina reads were binned according to index sequence. The data derived from the sequencing was processed using FastQC program (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). After initial trimming, we obtained the merged reads with FLASH v1.2.7 (<http://ccb.jhu.edu/software/FLASH/>) based on overlapping regions within paired-end reads (Magoč and Salzberg,

Following quality filtering, we generated a data set consisting of 15 102 475 high-quality and classifiable 16S rRNA gene sequences for further analysis. They were classified into different taxonomy using QIIME. The taxon abundance of each sample was generated into phylum, class, order, family, and genera levels using mainly database of RDP, aided by Greengene databases. At the phylum level, Proteobacteria and Firmicutes were the most dominant bacterial phyla, which consistently occurred in bacterial community of honeybees (Disayathanoowat *et al.*, 2012; Geng *et al.*, 2013). At the genus level (Fig. 1), *Kingella* of the Beta-Proteobacteria was the most abundant genus, and the amount of *Lactobacillus* was the second.

The DNA sequences of PCR products were



3.3 Effects of the sublethal doses of imidacloprid on the midgut bacterial composition of *A. mellifera ligustica*

The four feeding treatments were 0.005 mg/L imidacloprid syrup, 0.015 mg/L imidacloprid syrup, 0.045 mg/L imidacloprid syrup, and pure sugar syrup with 0.3% acetone. The histograms of the dominant midgut bacterial genera were generated from adult honeybee samples collected 3, 6, 9, 12, and 15 d after feeding on syrups with different concentrations of imidacloprid and pure sugar syrup (Fig. 2). In order to explore how the sublethal doses of imidacloprid affect on the composition of midgut bacteria, the composition of dominant midgut bacterial genera were analyzed for statistical significance among the four treatments in the same period by performing a *T*-test, and no significant differences were observed ($P > 0.05$). This result suggests that the sublethal doses of imidacloprid had no effect on midgut bacterial composition.

3.4 Effects of the sublethal doses of imidacloprid on the midgut bacterial structure of *A. mellifera ligustica*

The resultant high-quality sequences were clustered to operational taxonomic units (OTUs) at the cut-off of 97% similarity using UCLUST v1.2.22, then were used to calculate the Rarefaction curves, sample coverage and richness estimators in QIIME platform, and these histograms of Shannon estimator were constructed using QIIME toolkit (Fig. 3), providing a clear visualization of the relationships among the different sample groups. The richness estimators, including Shannon, *chaol*, and ACE, were analyzed for statistical significance among the four treatments in the same period by performing a *T*-test, and no significant differences were observed ($P > 0.05$). This result suggests that the sublethal doses of imidacloprid had no effect on midgut bacterial structure.

4 DISCUSSIONS

Effects of neonicotinoid pesticides on honeybees have been most widely studied, and their adverse effects on foraging behavior (Decourtye and Devillers, 2010), learning performance and memory (Decourtye *et al.*, 2011), motor activity (Lambin *et al.*, 2001), and olfactory capacity (Hassani *et al.*, 2005) have been well analyzed. Some metagenomic and experimental studies suggest that gut bacteria of honeybees play a major role in neutralization of dietary toxins, nutrition, and in defense against

pathogens (Engel *et al.*, 2012). The possibility of using the gut bacterial community as an indicator of honeybee health was discussed (Crotti *et al.*, 2013). However, the effects of the neonicotinoid pesticides on gut bacterial communities of honeybees have not been investigated yet. In this study, the effects of the sublethal doses of imidacloprid on the midgut bacterial communities of *A. mellifera ligustica* were first investigated under laboratory conditions. But no significant differences were found in midgut bacterial structures and composition among adult honeybees fed on syrups with different sublethal doses of imidacloprid (0.005, 0.015, and 0.045 mg/L) and pure sugar syrup with 0.03% acetone as a negative control group under the laboratory conditions. Moreover, the samples were collected at different time (3, 6, 9, 12, and 15 d), and no obvious differences were observed.

In this study, we used Illumina next-generation sequencing which is one of the non-culture based methods, to analyze the midgut bacterial community of the honeybees. The microbial community can be evaluated with high accuracy, low cost and short time by using the new sequencing technologies. The new deep sequencing methods provide a convenient platform for characterizing the composition of the animal microbiota. Furthermore, compared with earlier culture-based methods, using these methods can retrieve the most abundant members, so these new techniques have been widely used in analyzing the animal microbiota. Also the non-culture based methods have been used in characterizing the honeybee microbiota since 2003 (Jeyaprakash *et al.*, 2003), and recently these techniques have been applied in more and more experiments on honeybee microbiota (Cox-Foster *et al.*, 2007; Engel *et al.*, 2012; Martinson *et al.*, 2012).

The dominant midgut bacterial taxa of *A. mellifera ligustica* detected in this study were β -, γ -Proteobacteria and Firmicutes, consistent with the previous reports on the intestinal microbiota diversity of *A. mellifera* (Martinson *et al.*, 2011; Disayathanoowat *et al.*, 2012; Geng *et al.*, 2013). This result supports the previous opinion that honeybees have a co-evolved symbiotic relationship with a few specific bacterial groups once again (Martinson *et al.*, 2011). However, the diversity found was relatively low as compared with these studies. Furthermore, in this study, members of the β -Proteobacteria were found to be the most common group of bacteria in honeybee midgut, which was contrary to some studies (Babendreier *et al.*, 2007; Geng *et al.*, 2013). Geng *et al.* (2013), for example,

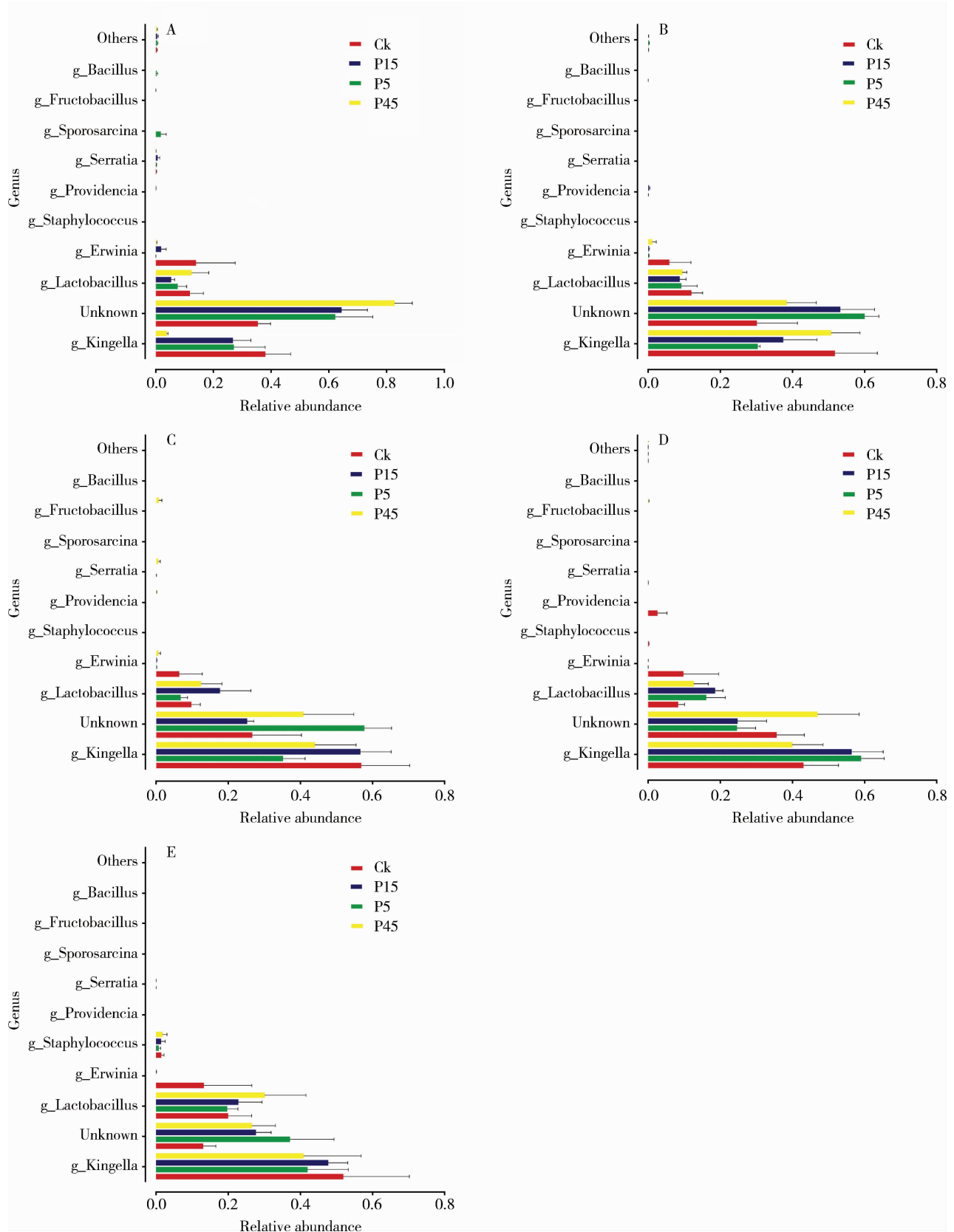


Fig. 2 Relative abundance of the dominant midgut bacterial genera in the midgut of *Apis mellifera ligustica* adults after exposure to imidacloprid for different time

A – E: Exposed to imidacloprid for 3, 6, 9, 12, and 15 d, respectively. Ck: Pure sugar syrup with 0.03% acetone; P5: 0.005 mg/L imidacloprid syrup; P15: 0.015 mg/L imidacloprid syrup; P45: 0.045 mg/L imidacloprid syrup.

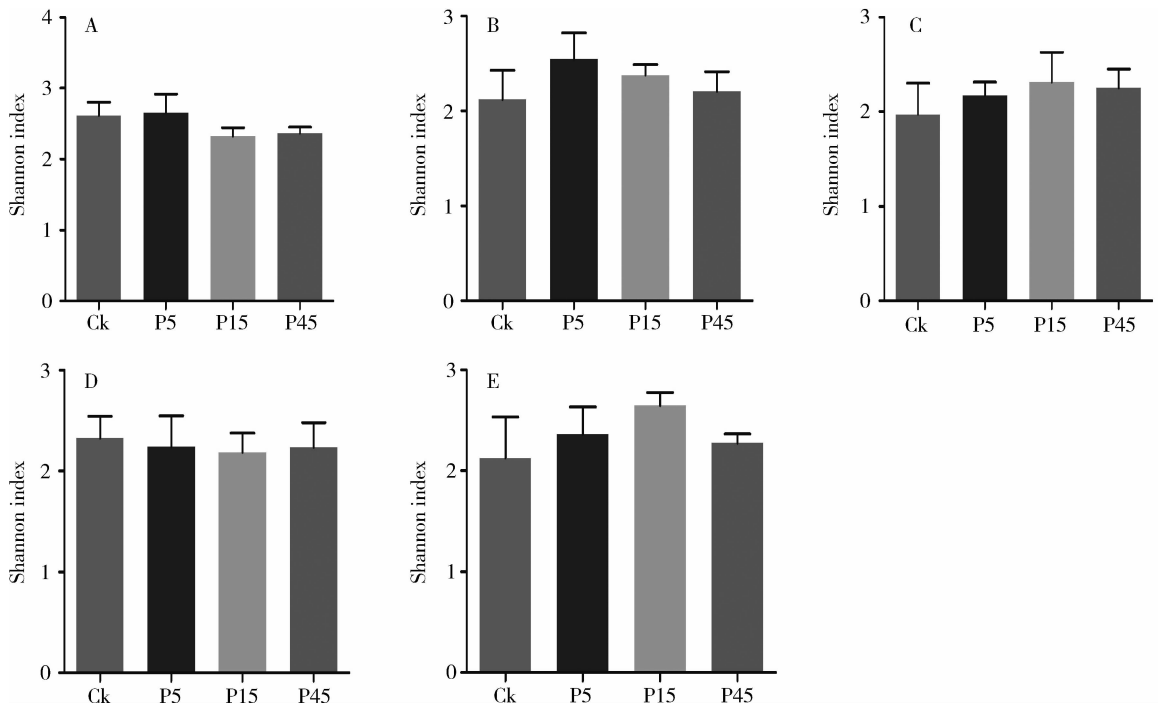


Fig. 3 Shannon index of bacterial diversity in the midgut of *Apis mellifera ligustica* adults after exposure to imidacloprid for different time calculated using QIIME toolkit

A – E: Exposed to imidacloprid for 3, 6, 9, 12, and 15 d, respectively. Ck: Pure sugar syrup with 0.03% acetone; P5: 0.005 mg/L imidacloprid syrup; P15: 0.015 mg/L imidacloprid syrup; P45: 0.045 mg/L imidacloprid syrup.

reported that γ -Proteobacteria was the most common group of bacteria in honeybee midgut. *Lactobacillus* is a major diversity genus in this study, which is in accordance with the previous studies (Vásquez *et al.*, 2012; Disayathanooat *et al.*, 2012). Increasing evidence shows that *Lactobacillus* plays a significant role in maintaining and improving honeybee health (Evans and Lopez, 2004).

Our study provides an insight into the potential risk of imidacloprid on the bacterial diversity in the midgut of *A. mellifera ligustica*. It concludes that sublethal doses of imidacloprid do not impact the bacterial diversity in the midgut of honeybees (*A. mellifera ligustica*) under laboratory conditions. Therefore, total bacterial community structures may not be a sensitive indicator for providing evidence for the impact of pesticide on honeybees at sublethal levels.

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亚致死剂量吡虫啉对意大利蜜蜂 中肠细菌群落的影响

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摘要:【目的】在实验室条件下,研究亚致死剂量吡虫啉对意大利蜜蜂 *Apis mellifera ligustica* 中肠细菌群落的影响, 以期为杀虫剂的生物安全性评价体系提供理论依据。【方法】分别用 3 种不同浓度(0.005, 0.015 和 0.045 mg/L) 的吡虫啉蔗糖溶液以及含丙酮(0.03%)的糖水(空白对照)在实验室内饲喂刚出房的意大利蜜蜂,在饲喂 3, 6, 9, 12 和 15 d 时分别取样。提取中肠细菌基因组 DNA,采用下一代高通量测序技术对 4 个处理组蜜蜂中肠细菌群落进行检测分析。【结果】细菌序列分析表明,意大利蜜蜂中肠细菌主要属于变形菌门(Proteobacteria)和厚壁菌门(Firmicutes),同一时间内各处理组间主要细菌数量差异不显著($P>0.05$),同时细菌群落多样性指数差异也不显著($P>0.05$)。【结论】在实验室条件下亚致死剂量吡虫啉对意大利蜜蜂中肠细菌群落结构影响不显著。

关键词: 意大利蜜蜂; 生物安全性; 吡虫啉; 亚致死剂量; 肠道菌群; 下一代测序

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